Crystallization of biological macromolecules for X-ray diffraction studies

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Advances in the crystallization of biological macromolecules have come not only from the application of biochemical, molecular biological and immunological principles and techniques, but also from continued efforts to understand the crystallization process. Developments in crystallization methodologies, protocols, and reagents are also facilitating crystallization efforts.

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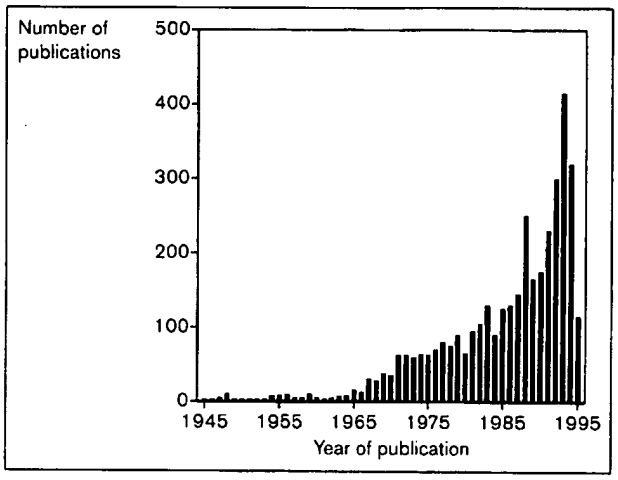
Abbreviations

2D	two-dimensional
3D	three-dimensional
PEG	polyethylene glycol

Figure 1



The total number of publications in Version 3.0 of the Biological Macromolecules Crystallization Database [2] for each year of publication. The number of references is directly correlated with the number of crystal entries contained in the database.

Introduction

The three-dimensional structure determination of a biological macromolecule by X-ray crystallographic techniques requires the production of large single crystals. Establishing the correct conditions for the crystallization of a particular biological macromolecule is an empirical process that typically uses techniques and reagents that have proven successful in other cases. The accelerating growth of the Brookhaven Protein Data Bank [1] and the growing number of entries in the Biological Macromolecule Crystallization Database [2] (see Fig. 1) are two measures of the usefulness of these approaches.

Despite the many successes of crystallization studies, however, our knowledge of macromolecular crystal-growth processes remains rather limited. Recently, various experimental techniques have been newly applied to the investigation of the crystallization process, furthering our understanding of how biological macromolecules crystallize (for recent reviews, see [3•,4•]).

This article reviews recent results from studies directed towards obtaining crystals of biological macromolecules for diffraction experiments, and from studies aimed at understanding many aspects of the crystal-growth process. Many recent advances in crystallization have come from the application of biochemical, molecular biological, and immunological principles and techniques. The importance of macromolecular purity and solubility remains the focus of crystal-growth studies, and the application of a variety of techniques has advanced our understanding of crystal nucleation and growth. Recent crystal-growth studies in space have continued the trend towards investigating the influence of gravity on crystal quality and the crystallization process. In addition, modifications of standard methods and strategies for crystallization, together with new crystallization techniques, offer novel ways to approach the crystallization of a particular biological macromolecule. Though the focus of this review is the crystallization of soluble biological macromolecules, the principles and techniques described here are applicable to membrane proteins also (for a recent review, see [5]).

Sample selection, purity, and solubility properties

In the early years of protein crystallography, it was repeatedly discovered that changing the biological source or limited proteolysis of a macromolecule used in the crystallization experiments would often produce (or improve) crystals suitable for diffraction studies. Thus, it was discovered that small changes in the amino acid sequence are often sufficient to influence favorably the crystallization behavior of a protein. Systematic protease treatment of proteins still provides a route towards improving the diffraction quality of crystals [6].

Site-directed mutagenesis

The powerful tools of recombinant DNA technology provide the means to make specific changes in a

protein sequence to improve its solubility and hence crystallizability. Success in the crystallization and structure determination of HIV integrase [7] provides perhaps the best example. Attempts at crystallizing this protein in many laboratories were carried out before anyone had employed a systematic approach of improving the crystallizability by changing hydrophobic residues to hydrophilic residues. This has been extended in studies of a protein–nucleic acid complex by combining alterations of the protein sequence with the variation of the nucleic acid sequence of an RNA hairpin to optimize the production and diffraction quality of crystals [8].

Site-directed mutagenesis also offers a mechanism of investigating the role of specific residues in crystal lattice contacts and of introducing new interactions at lattice contact points. For example, a lattice contact in crystals of glutathione reductase from *Escherichia coli* was strengthened by the addition of a new interaction introduced by a double mutation, Ala86His and Ala90Tyr [9]. The diffraction quality of the crystal was not improved but the crystals of the variant grew 40 times faster than the wild-type crystals. Another recent example is the alteration of the two-dimensional (2D) crystallization behavior of horse apoferritin [10]. Two residues, Asp84 and Gln86, known to interact with Cd^{2+} ions essential for crystal-growth, were replaced with serine. Two new 2D crystal forms that grow in conditions independent of Cd^{2+} ions were observed.

Fusion proteins

Several investigators have proposed the use of fusion protein systems to aid in difficult peptide or protein crystallization problems [11–13]. Privé and co-workers [11] have proposed the fusion of carrier proteins that can be introduced into an internal position of a target protein to increase the solubility and hence its crystallizability. The carrier protein requirements include: solubility, a single compact domain, crystallizability, N- and C-termini close together on the surface of the structure, no disulfide bonds, easily cloned and expressed, larger than the target protein, a measurable enzymatic activity or color, and purification by affinity chromatography. Privé and co-workers [11] introduced the *E. coli* cytochrome b_{562} into lactose permease, a membrane protein; however, the method should prove useful for other proteins with low solubility or stability.

Two other studies involving crystallization and structure determination of fusion proteins used other carrier proteins, chicken egg-white lysozyme [12] and *Shistosoma japonicum* glutathione S-transferase [13]. The initial phases in the crystal structures were obtained by the molecular-replacement technique. Both peptides, the human fibrinogen γ-chain C-terminal segment (residues 398–411) and a hexapeptide conserved epitope of HIV-1 gp41, were fused to the C-terminus of the carrier protein, lysozyme and glutathione S-transferase, respectively. In both cases, the peptide conformations are stabilized not only by interactions with the carrier protein but also by interactions with symmetry-related carrier molecules. Although, in both cases, the question remains whether or not the conformation of the peptide is similar to that found in the natural protein, this technique holds the promise of providing structural information for this class of small, difficult-to-crystallize structures.

Complexes with antibody fragments

Since the report of the first high-resolution crystal structure of an antigen–antibody complex, an Fab bound to hen egg-white lysozyme [14], many complexes of antigens and antibody fragments have followed, revealing the structural principals that govern antibody–antigen interactions. This immunological strategy has also yielded structures of biological macromolecules that have otherwise proved difficult to crystallize. The use of Fab and Fv in the crystallization and the structure determination of biological macromolecules and viruses has been reviewed recently [15•]. The combination of the immunoglobulin fragment and the antigen will often drastically change the solubility properties and stability of the antigen, making it more amenable to crystallization. A novel study that successfully crystallized a complex of chicken egg-white cystatin and a recombinant Fab fragment was enhanced by generating three variant Fabs with differing constant domains [16]. The antibody fragment structure is often easily determined by molecular-replacement methods and can provide the initial phases that leads directly to a complete structure solution of the complex.

Macromolecule purity

The effects of purity on the crystallization of chicken egg-white lysozyme continue to be investigated [17–19]. These studies support much earlier work that indicated that the purer the biological macromolecule the better the chance of producing suitable crystals for diffraction studies and of reproducing the crystals from preparation to preparation of the macromolecule.

Analysis of impurities in commercial preparations [17,19] detected varying amounts of contamination by other proteins present in chicken egg white. Dynamic light-scattering measurements indicate aggregation of lysozyme and contaminating proteins occurs, and it was speculated that this may lead to heterogeneous nucleation leading to the formation of ill-shaped microcrystals [17].

Ewing and co-workers [19] identified in commercial preparations of lysozyme three classes of impurities that influence the crystallization behavior: contaminating proteins, small molecules, and heterogeneous forms of the protein. Of these, the heterogeneous forms of lysozme appeared to have the greatest perturbing influence on crystallization behavior.

A new study on the growth of chicken egg-white lysozyme contaminated with turkey egg-white lysozyme, a protein with 95% sequence identity, shows crystal nucleation inhibition and morphology changes [18]. This study contrasts growth studies in solution with those in agarose and silica gels. A higher concentration of contaminant is tolerated in the gel experiments than in the solution experiments. The findings show that lower concentrations of contaminants affect nucleation whereas higher concentrations influence crystal-growth morphology.

Aggregation state and solubility

The crystallizability of a biological macromolecule is dependent upon its solubility properties. Static and dynamic light-scattering measurements have been used to examine the aggregation state and solubility of biological macromolecules [20]. A correlation between the presence of a monodisperse species of biological macromolecules in the crystallization solution and crystal-growth has been observed. Recent studies of lysozyme solutions showing that the hydrodynamic interactions of the protein decrease significantly with increasing salt concentration agree with earlier studies [21]. As the salt concentration is increased, the interactions between protein molecules change from repulsive to attractive. These new studies suggest that salt ions are binding to the surface of the protein, or that their presence changes the dielectric constant of the medium.

The effects of different anions on the solubility of an acidic protein *Hypoderma lineatum* collagenase, which has a pI of 4.1, was measured at pH 7.2 [22•]. The anions of ammonium salts were ranked as $HPO_4^{2-}/HPO_4^{-} > SO_4^{2-} > citrate^{3-}/citrate^{2-} >> Cl^{-}$ in their ability to decrease the solubility of this protein. This is in agreement with the ancient studies of Hofmeister [23], who found similar results with the ability of anions to precipitate chicken egg-white proteins, but in complete contrast with findings for basic proteins such as lysozyme [24]. If these findings can be generalized, the iosoelectric point of the biological macromolecule may dictate the choice of salts for crystallization trials.

A comparison of the solubility of chicken egg-white lysozyme in solutions with either H_2O or ^2H_2O has produced interesting results [25•]. The studies show that lysozyme is 1.3 times more soluble in ^2H_2O than in H_2O. The higher solubility, and thus the higher supersaturation, may prove useful in crystal-growth studies if this observation holds true for other biological macromolecules.

Fundamentals of crystal growth

The crystal-growth process for biological macromolecules is no different from that for other substances. The process can be divided into discrete stages, nucleation, crystal-growth, and cessation of growth. Each of these stages is dependent upon the solution properties of the biological macromolecule and the state of the system.

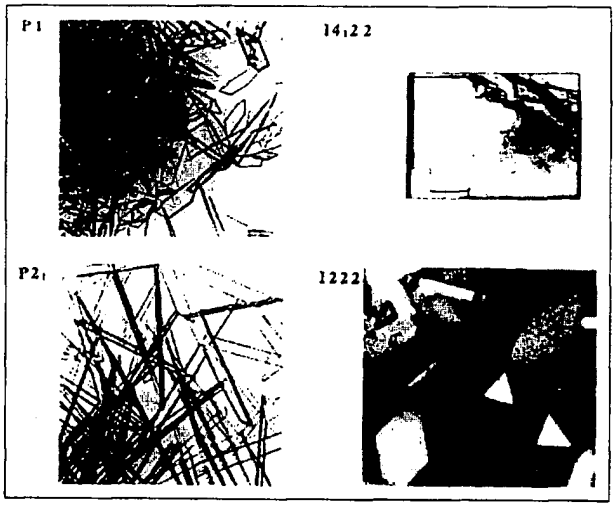
The study of aspects of the crystal-growth process has become a focus of many laboratories throughout the world. This work has established the general principle that the conditions that favor nucleation are different from those required to sustain crystal-growth.

Nucleation and epitaxial growth

Two common problems are associated with nucleation: either no nucleation occurs, and hence no crystals are produced; or too many nuclei form, producing a shower of microcrystals that may never grow large enough for diffraction studies. The use of heterogeneous nucleants to initiate nucleation and the use of filtration to reduce nucleation are two approaches to solving these problems.

Recent work has highlighted a promising method of growing crystals by epitaxial nucleation on a charged lipid layer [26]. In the original application of this method, biotinated lipid layers were used to produce 2D crystals of streptavidin that nucleated the growth of three-dimensional (3D) crystals of streptavidin [27]. The approach was then broadened to show that charged lipids could be used instead of lipids with a bound ligand (see Fig. 2) [28••].

Figure 2



The morphology of four crystal forms of apostreptavidin grown in hanging drops. The $I4_122$ crystal shown was grown on a coverslip using biotinylated lipids to nucleate growth. The pictures were all taken at the same magnification; the width of each picture is equivalent to 2 mm. Reproduced with permission from [28••].

This method is, in some respects, an extension of the growth of protein crystals by epitaxial nucleation on the surfaces of minerals [29]. In this case, non-specific electrostatic interactions of the protein at the lipid surface result in the concentration of the protein in that region and thus an increase in the possibility of nucleation.

The crystals grow very rapidly and at lower protein concentrations than those normally used. For the growth of streptavidin crystals, the protein concentration can be as low as 10 μM [28••].

In another experiment with streptavidin, 2D crystals were grown using a monolayer of an iminodiacetate-Cu(II) lipid [30••]. The surface His87 was shown to bind to the Cu(II) ion. Mutants His87Ala and His127Cys were used to demonstrate the specificity of the binding. It was proposed that the method would be general for proteins with solvent-exposed histidine residues and could be expanded to include proteins that were specifically mutated to display surface histidines. The further expansion to altering the conditions to allow 3D crystal-growth seems imminent.

Several techniques for the control of nucleation have been proposed [31,32]. In these studies, filtration of the protein solution was found to reduce nucleation. By varying the exclusion size of the filters, it was shown that the number of nuclei decreases with decrease in pore size. Nucleation was also controlled by placing the droplets under parafilm oil, reducing the contact region of the supporting vessel, and by changing the temperature once nucleation has occurred to favor crystal-growth [31]. The use of gelled media in nucleating studies reduced the nucleation events, whereas the aging of a protein solution was shown to have little or no effect on nucleation [32].

Crystal growth kinetics

Techniques for studying the kinetics of crystal-growth have been reviewed recently by McPherson and co-workers [3•]. Simple visual microscopy, Michelson and Mach–Zehnder interferometry, and atomic force microscopy are all being successfully employed to study crystal-growth kinetics and other phenomena. Visual microscopy has been employed to measure growth rates of tetragonal chicken egg-white lysozyme [33,34] and jack bean concanavalin A [35].

The use of Michelson interferometry, which has also been applied to tetragonal lysozyme, is providing insight into the crystal-growth process [36•,37–39]. In these studies, the growth of a particular crystal face, such as the (101) face of tetragonal lysozyme, is measured optically by observing the changing interference pattern as a function of time while varying a crystallization parameter. The crystal-growth mechanism of tetragonal lysozyme was shown to change at a critical saturation point [36•]. These studies have led to recommendations for conditions for growing larger and more perfect crystals by using macromolecules of the highest purity, by growing crystals at elevated temperatures, and by growing crystals at a precipitant concentration at the low end of the range of concentrations that maintain supersaturation.

Further experimental and modeling studies [37,38] have shown that the crystal-growth interface attains a convex shape that increases with crystal size and growth rate with pure protein. In contrast, the crystal-growth interface attains a concave shape when impurities are present. This effect is also dependent on crystal size, indicating its relationship to transport-induced impurity non-uniformities at the crystal-growth interface. Further studies on the influence of impurities on the crystal-growth process of tetragonal lysozyme have shown that the presence of < 1% protein impurities perceptibly alters the growth kinetics and compositional uniformity of the crystals [39].

Gravity

The presence of the gravitational field and its influence of the movements of molecules in solution is a constant component of all laboratory crystallization attempts. Studies carried out in space since the early 1980s on the US NASA Space Shuttles, the Russian *MIR* Space Station and other unmanned platforms have been investigating the influence of gravity in macromolecular crystal-growth (for a recent review, see [40]). In several instances, crystals grown in the early studies in space did diffract to measurably higher resolution than those grown in the laboratory, but in other cases no improvement was evident. New reports document improvements in the diffraction quality for both virus and protein crystals [41–43]. Even though the crystals sometimes diffract to higher resolution than the earth-grown counterparts [41], the crystallization process remains subject to all of the other earth-bound variables, including the choice of method, i.e. the vapor diffusion method versus liquid–liquid diffusion method [42]. In one recent example, crystallization studies of apocrustacyanin C1 grown in space and in the laboratory indicate that the crystals grown in microgravity diffract to higher resolution than those grown in the laboratory control experiments in the same reaction vessels [43]; however, the space-grown crystals did not diffract as well as those obtained using another technique.

The diffraction quality or limiting resolution of the data from a crystal is an important parameter for assessing the quality of a single crystal, but another measure of perfection is the mosaicity. Snell and co-workers [44•] have shown that the mosaicity of crystals of lysozyme grown on two different space flights offered a four-fold improvement over the mosaicity of crystals grown in control laboratory experiments. This difference in mosaic spread provides further evidence that the force of gravity is influencing the crystal-growth process in the laboratory, at least partly through gravity-induced convection and altered solution-transport properties.

Crystallization techniques and reagents

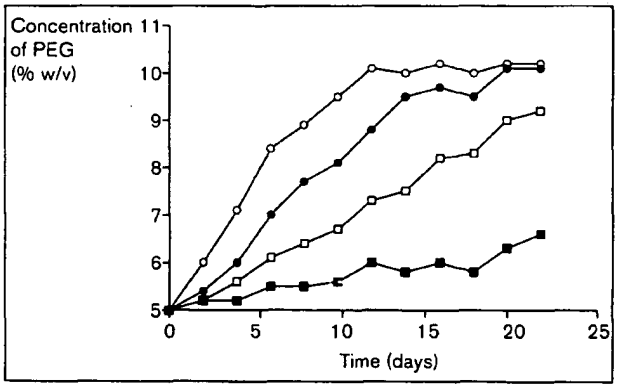
The methods used in macromolecular crystal-growth are continually being refined. The most frequently used method is vapor diffusion with either hanging or sitting

drops. Here, we review reports of attempts to overcome specific problems ranging from how to induce more proteins to crystallize to how to prevent crystals from sticking to the coverslips used to support the drops.

Vapor diffusion and polyethylene glycol

Detailed studies of the vapor-diffusion technique and the effect of polyethylene glycol (PEG) on water–vapor equilibration have yielded some quite surprising results [45•]. Using PEG8000, for example, in a 24 µl droplet at an initial concentration of 5.0% (w/v) with twice the concentration in the reservoir, 12 weeks are required to reach equilibrium at 293 K. The addition of 200 mM sodium chloride to the droplet and reservoir decreases the equilibration time to 2 weeks (see Fig. 3). The results are a consequence of the non-ideality of aqueous PEG solutions. Understanding these interactions can add yet another set of options when planning and refining crystallization experiments.

Figure 3



Equilibration of vapor-diffusion experiments containing PEG solutions with NaCl. The concentration of PEG in the droplet is plotted as a function of time for equilibrations involving PEG solutions with varying quantities of NaCl. The equilibrations were carried out at 293 K using 24 µl sitting droplets with 1 ml reservoirs. All reservoir concentrations were 10%(w/v) PEG8000, plus (■) 0 mM, (□) 100 mM, (●) 200 mM, and (○) 400 mM NaCl. The initial droplet concentrations were half that of the reservoir. Each point is the mean of six observations. Adapted with permission from [45•].

Other studies have focused on determining the concentration of salt that exhibits an equivalent vapor pressure as a given concentration of PEG. The lesson is that relatively low salt concentrations are equivalent to relatively high PEG concentrations [46•]. For example, 45 mM ammonium sulfate, 60 mM sodium chloride or 100 mM magnesium sulfate heptahydrate in the reservoir all have the same vapor pressure of water as 15% (w/v) PEG8000 in the droplet.

In yet another series of experiments, the residual air pressure in the vapor chamber for PEG solutions was studied [47]. It was found that a decrease in pressure leads to an increase in the rate of equilibration, and it was suggested that appropriate alteration of the pressure in the vapor chamber might enable the time course of the crystallization to be controlled.

One problem encountered with sandwich drops or sitting drops is that the crystals can sometimes adhere quite strongly to the support surface. In response to this problem, the plug drop has been suggested [48]. A 1 cm length of 4 mm siliconized glass tubing is epoxyed to a coverslip, and a macromolecule solution is then placed in the tube, forming a plug of liquid. This is then equilibrated against a reservoir as usual but the crystals fall to the liquid–air interface as they grow instead of onto the drop-support surface.

Shaped crystals

Crystallography can be applied to the study of kinetics of macromolecular reactions by conducting experiments where substrates are diffused through the crystal lattice. Growing crystals that completely fill the cross-section of a capillary has been suggested as a way of simplifying these experiments [49•]. Gel-acupuncture under isothermal conditions is used to limit the number of crystals to less than three per capillary; then the crystals are allowed to grow and assume the morphology of the inside of the capillary. Using this technique, chicken egg-white lysozyme crystals with circular cross-sections of 0.2 and 0.5 mm diameter have been grown and shown to diffract as well as crystals grown by other methods.

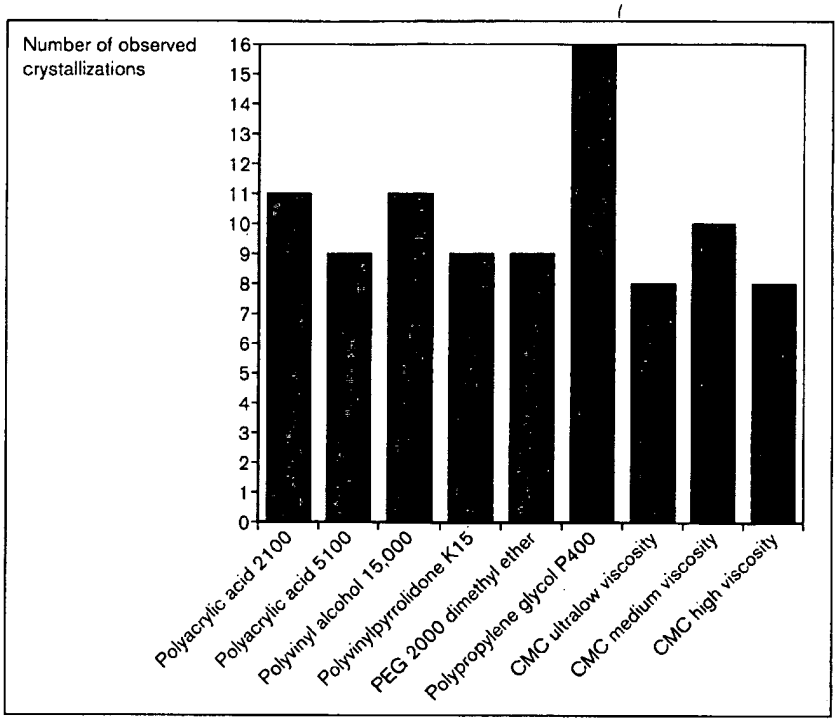
Crystallization reagents

The search continues for a magic crystallizing agent. No one has claimed to have found it, but some new compounds have been offered for consideration when screening for crystals. Nine different commercially available soluble polymers have been tested with a series of biologically relevant molecules that includes 24 proteins, three viruses and two small biologically relevant molecules [50••]. With a coarse screen of crystallization conditions, all three viruses, both small molecules and 14 of the proteins crystallized. The success rate and compounds are shown in Figure 4.

Glycerol is often used in crystallization mixtures, as an additive to the macromolecule solution, a crystallizing agent, and a cryoprotectant. In a review of glycerol and other polyols by Sousa [51], it is pointed out that glucose, sorbitol, sucrose, glycerol and most other polyols increase protein thermal-denaturation temperatures. Consequently, it is postulated that the inclusion of the agents in the crystallization mix can help suppress conformational flexibility and stabilize the protein against denaturation.

Figure 4

Crystallization of proteins with novel soluble polymers. The graph records the number of proteins successfully crystallized (out of a battery of 24 proteins) for each of nine different soluble polymers. Reproduced with permission from [50••].



In another report of crystallization additives, short-chain phospholipids were used to lower the solubility of crambin and retard crystal-growth in the normally fastest growing direction [52•]. For crambin, the most effective additive, phosphotidylcholine, has a size comparable to the proposed binding site. In this case, the inclusion of phospholipids reduced the protein concentration needed for crystallization by 30–60-fold.

Crystallization strategies

As mentioned in the introduction, the search for crystallization conditions is an empirical process that relies on our knowledge of what has worked in the past for other biological macromolecules. Surprisingly, no generally accepted strategy has emerged, even though many suggestions have been put forth [53], with the exception perhaps of the implementation of a fast screen procedure [54] (see below). The search for solution conditions that produce crystals suitable for diffraction studies requires that parameters such as pH, protein concentration, temperature, ionic strength, etc. be sampled over a wide range of values. Once crystal-growth conditions are found, the parameters are optimized to produce crystals of suitable size and quality for diffraction studies. Recent suggestions for sampling and optimizing conditions found in this multidimensional space [55–57], along with new fast screens [54,58], have been put forth.

Sampling techniques

Carter and Carter [59] proposed the use of the incomplete factorial method to reduce the number of experiments that one needed to carry out in the crystallization discovery process. This method was subsequently refined to develop a more systematic method [60]. Recently, a similar procedure that has had measurable success has been reported [55]. The method starts with the initiation of experiments based on random sampling of crystallization parameters followed by a quantitative assessment of the results. The parameters are then assigned weights based on the results. Further experiments are then based on this weighted parameter set. This is repeated until crystals suitable for diffraction experiments are obtained.

In an interesting study, Carter and co-workers [56] employed quantitative analysis of full-factorial crystallization experiments to analyze the perturbation of a protein structure, that of tryptophanyl-tRNA synthetase. The study reveals that crystallization parameters including ligands alter the protein conformation, resulting in changes in crystallization behavior. Indeed, different stages of catalysis are trapped in different crystal forms depending on the environment and hence the conformation of the protein.

After microcrystals of a biological macromolecule are discovered, and the optimization of parameters such as

precipitant, pH, temperature, etc., does not produce crystals, the specific sampling of pH in small increments has been found to produce crystals suitable for diffraction studies [57]. This procedure samples pH at intervals of 0.05 units over the range of pH that microcrystals are observed. Five different sets of experiments using five different buffers over the pH range are recommended.

Fast screening

Many recent successes in the crystallization of biological macromolecules are the results of implementing the fast-screen technique first popularized by Jancarik and Kim [54]. Fast screens use premixed solutions that have frequently produced crystals for setting up crystallization experiments. A new fast screen for RNA was devised and used in combination with a second screen that varied the RNA sequence in hammerhead RNA constructs [58]. The RNA screen employs salts, organic reagents and several different molecular weight PEGs as precipitants, with a variety of salts as additives. One of six hammerhead RNA constructs crystallized in a form suitable for diffraction studies.

Conclusions

The continued demand for structural information of biological macromolecules has maintained the necessity for obtaining suitable crystals for diffraction studies. Indeed, the study of biological processes is offering up new challenges to structural biologists. Crystals of not only single components but also of macromolecular assemblies are required to discover the structural basis for function. The complexes include those composed of only proteins, and those composed of proteins and nucleic acids.

The fundamental problem of finding crystallization conditions for a new macromolecule or macromolecular assembly has not changed, but the tools for manipulating the systems that are under investigation to produce materials that crystallize have improved. The many successes in growing suitable crystals for diffraction studies coupled with the application of novel techniques have increased our understanding of the crystallization process. Yet further ingenuity (and luck) are often required to produce X-ray quality crystals of a specific material. Additional studies that will increase our understanding of the fundamental processes of crystal-growth are needed until a coherent picture of the processes of crystal-growth is obtained.

Acknowledgement

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